Generation of human islet-specific regulatory T cells by TCR gene transfer.

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Abstract

Based on the success in animal models of type 1 diabetes (T1D), clinical trials of adoptive regulatory T cell (Treg) therapy are underway using *ex vivo* expanded polyclonal Tregs. However, pre-clinical data also demonstrate that islet-specific Tregs are more potent than polyclonal Tregs at reversing T1D. Translation of this approach into man will require methods to generate large populations of islet-specific Tregs which, to date, has proved to be a major hurdle. Here we demonstrate the feasibility of lentiviral-mediated T cell receptor (TCR) gene transfer to confer antigen specificity on polyclonal human Tregs. Targeting has been achieved using TCRs isolated from human islet-specific and viral-specific CD4+ T cell clones. Engineered T cells demonstrated expression of ectopically-delivered TCRs, resulting in endowment of cognate antigen-specific responses. This enabled antigen-specific suppression at increased potency compared to polyclonal Tregs. However, cells transduced with islet-specific TCRs were less responsive to cognate antigen than viral-specific TCRs, and in some cases, required additional methods to isolate functional antigen-specific Tregs. This study demonstrates the potential of TCR gene transfer to develop islet-specific Treg therapies for effective treatment of T1D, but also highlights that additional optimisation may be required to achieve its full potential.

Keywords:
Regulatory T cells
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1. Introduction

Type 1 diabetes (T1D) results from a profound dysregulation of the T-cell immune response. This is manifested by an expansion of pathogenic islet-specific T cells, leading to progressive destruction of pancreatic β-cells[1]. Populations of Tregs found in the periphery, including those expressing the transcription factor Forkhead Box Protein 3 (FOXP3)[2], are crucial in maintaining immunological tolerance. The fundamental role played by Tregs in controlling autoimmunity is clearly demonstrated by the human syndrome, 'Immune dysregulation, polyendocrinopathy, enteropathy, X-linked' (IPEX), where a loss-of-function mutation is present in the FOXP3 gene. This mutation leads to development of a range of autoimmune disorders, including T1D, in >80% of individuals by the age of 2. While it is now established that the frequency of Tregs in individuals with T1D is similar to that seen in control individuals[3, 4], several studies have shown that the functional ability of Tregs to suppress autologous effector T cells is significantly reduced in individuals with T1D. This abnormality is evident before clinical diagnosis, at the time of diagnosis and many years following onset of T1D[4-7]. Since defective Treg function appears to be central to the pathogenesis of T1D, it is logical to hypothesise that correction of this imbalance may slow or prevent disease progression. Consequently, strategies aimed at increasing the number or functional potency of Tregs constitute a major focus of clinical trial activity relating T1D.

One alternative option to therapeutically manipulate Tregs is the adoptive transfer of *ex vivo* cultured cells. This involves the isolation, expansion and/or invigoration of Tregs from an individual, followed by their re-infusion into the patient. This therapeutic approach has shown great success in animal models of transplantation and autoimmunity[8]. Clinical protocols have now been developed to generate large populations of human Tregs by magnetic bead enrichment or fluorescence activated cell sorting (FACS), followed by expansion *in vitro* in the presence of polyclonal stimuli and high concentrations of IL-2. Trials of Treg therapy are underway in several disease indications and have already shown significant clinical benefit in patients with Graft versus host

disease[9-11]. In T1D, two clinical trials have been performed demonstrating the feasibility and safety of this approach[12-14].

While polyclonal Treg therapy has now reached the clinic, data from mouse models of T1D have shown that adoptive transfer of antigen-specific Treg populations is more potent at controlling T1D[15-18] and may even reverse disease[15]. To translate antigen-specific Treg therapy into the clinical setting, protocols to generate large populations of antigen-specific Tregs need to be developed. One approach to achieve this is to selectively expand Tregs with the desired specificity by stimulating Tregs with antigen presenting cells (APC) bearing the appropriate epitopes on the cell surface. Whilst this approach has been used to generate populations of alloantigen-specific Tregs for use in the field of transplant tolerance[19], applying these protocols in T1D would prove difficult due to the very low frequency of islet antigen-specific Tregs in the blood. An alternative method for producing antigen-specific T cells is by TCR gene transfer. Feasibility of this approach has been demonstrated in both mouse and human Treg systems[20-22], but only using model antigen-specific TCRs (e.g. virus or tumor specific TCRs). By contrast, this has never previously been achieved using TCRs isolated from *bona fide* autoreactive or islet-specific human T cells.

In the present study we tested the ability of TCRs isolated from two islet-specific T cell clones[23] to re-direct the antigen specificity of polyclonal human Tregs and compared the function of these transgenic Tregs to those generated using TCRs directed against viral antigens. Following lentiviral-mediated transfer of TCR genes, we successfully demonstrated expression of islet-specific TCRs, which resulted in the re-direction of antigen specificity towards cognate islet autoantigens. Islet-specific transgenic Tregs were capable of suppressing CD4+ and CD8+ effector T cell proliferation in a manner that was potentiated by exposure to the appropriate islet autoantigen. Notably, the ability of the islet antigen-specific TCRs to induce Treg activation and mediate antigen-specific suppression was significantly lower when compared to levels of activation or suppression achieved using TCRs with specificity for viral antigens. These findings demonstrate the feasibility of this approach for the development of

antigen-specific Treg therapies for the treatment of T1D. They also raise questions regarding the optimal T cell populations to use as sources of autoreactive TCRs.

2. Materials and methods

2.1. Construction of TCR lentiviral plasmids.

To identify the nucleotide sequences of each TCR, RNA was extracted from T cell clones using the RNeasy total RNA extraction kit (Qiagen). First strand cDNA was produced by 5′RACE (SMARTer 5′RACE kit, Clontech) and cDNA for TCR α and β chains were amplified in separate reactions using a TCR−chain specific and 5′RACE primers (TRBC primer: 5′-GCTGACCCCACTGTGCACCTCCTTCCC-3′ TRAC primer: 5′-CCAGGCCACAGCACTGTTGCTCTTGAAGTCC-3′), using Phusion polymerase (New England Biolabs).

DNA products were ligated into the pGEM-T easy vector (Promega) and three independently isolated plasmids were sequenced for each TCR chain. Sequences were analyzed using the International Immunogenetics information system (IMGT)/V-QUEST[24, 25] to identify the allele usage of the TCR chain. Following identification of TCR gene sequences, a porcine teschovirus-1 (P2A) ribosomal skip sequence was constructed to link TCR α and β coding sequences using overlap extension PCR. AvrII and SalI restriction sites were introduced to the 5′ and 3′ of the insert for ligation into the pELNSxm lentiviral backbone, which also encodes for mCherry as a fluorescent reporter.

2.2 Production of lentivirus and transduction of Jurkat cells.

To produce lentivirus (LV), 293T cells were co-transfected with the following plasmids: TCR expression plasmid, and pCRV-1[26] encoding viral *gag/pol* and vesicular stomatitis virus G (VSV-G) viral envelope protein. Vector was collected 72 hours later, titrated and stored at -80˚C. Wild-type Jurkat cells (containing endogenous TCR) or J76 Jurkat cells (lacking an endogenous TCR) were transduced with LV by spin inoculation and transgene expression assessed by flow cytometry after 72 hours.

2.3 Production and expansion of transgenic Treg populations.

Peripheral blood mononuclear cells (PBMCs) were isolated from 100 ml fresh blood from HLA-compatible donors. PBMCs were stained with anti-human CD4-APC (BioLegend), anti-human CD25-PE (BD Biosciences) and anti-human CD127-Brilliant Violet 421 (BioLegend). Single lymphocytes were identified based on forward and side scatter parameters and $CD4+CD25^{hi}CD127^{lo}$ cells were isolated as the Treg population using a BD FACS Aria III flow sorter and FACSDiva software (BD Biosciences). Isolated Tregs were activated with a 1:1 ratio of anti-CD3/CD28 coated microbeads (Dynal, Thermo Fisher) in round-bottomed 96 well plates at $5x10^4$ cells/well in 200 µl Treg media (X-VIVO 15 media (Lonza) supplemented with 5% human serum (Sigma), 1 % pen/strep/fungizone, 1,200 IU/ml IL-2 (Proleukin Novartis) and 125 ng/ml Rapamycin (Rapamune, Pfizer)). Rapamycin was included in cultures to promote the selective expansion of FOXP3+ Tregs [27, 28]. After 48 hours, Tregs were harvested and spin inoculated at a multiplicity of infection of 4 TU/cell, washed and cultured in flat-bottomed 96 well plates, splitting cells as required. After 8-12 days, transduced Tregs were identified by expression of mCherry red fluorescent protein and isolated by flow sorting before being re-expanded as described above for 1-2 cycles. Following the final expansion cycle, Tregs were incubated in media lacking IL-2 and Rapamycin for 48 hours prior to phenotypic and functional assessment. The purity of expanded Tregs was assessed by flow cytometry after staining with anti-CD4-APC, CD25-PE, and FOXP3-V450 using the eBioscience FOXP3 staining kit.

2.4 Assessment of antigen specificity of transgenic T cell populations.

Initial testing of TCR function measured upregulation of the early activation marker CD69 on Jurkat cells. Briefly, Jurkat cells were incubated with HLA-compatible Epstein Barr virus (EBV) transformed B cells labelled with CellTrace violet (Life Technologies) at a ratio of 1:2 in round-bottomed 96 well plates at 37˚C, in the presence or absence of cognate peptide (listed in table 1) or CytoStim (Miltenyi) as a positive control. After 16-18 hours, cells were harvested, stained with anti-CD69-APC (BioLegend) and analyzed by flow cytometry. Assessment of TCR function in Tregs used a similar protocol, except

CellTrace Violet labeled PBMCs were used as the source of APC at a ratio of 10:1 with transgenic Tregs.

2.5 Antigen-specific suppression assays.

CellTrace Violet labeled PBMCs from HLA-compatible donors were stimulated with recall antigens Pediacel (pentameric vaccine, Sanofi Pasteur) or Agrippal (Influenza vaccine, Novartis) and transgenic ($mCherry^+$) Tregs were added at various ratios. After 7 days, cells were harvested, stained with anti-CD4-APC and anti-CD3-PE-Cy7 antibodies and 7-AAD and were analyzed by flow cytometry.

2.6 Data analysis.

Flow cytometry data was collected using a BD FACS Canto II and analyzed using FlowJo v10 (Tree Star Inc.). Graphs were constructed and statistical analysis performed using GraphPad Prism (GraphPad Software).

3. Results

3.1 Functional analysis of transgenic TCRs in Jurkat cells.

We isolated and sequenced TCR genes from two islet-specific CD4+ T-cell clones, namely RAR5.3 (IA2-specific) and MHB10.3 (insulin-specific). As a control, we also cloned TCR genes from an influenza-specific CD4+ T cell clone (HA307). Single functionally rearranged TCR α and β chain nucleotide sequences were identified for each T cell clone which are summarised in table 1 along with TCR allele usage, peptide specificity and HLA- restriction.

Equimolar expression of both TCR genes was obtained using a multicistronic lentiviral expression vector in which mCherry red fluorescent protein provided a convenient marker of transduction efficiency (Figure 1A). Recombinant LV was used to infect J76 TCR negative Jurkat cells, resulting in >90% transduction and expression of transgenic TCR by >90% of transduced cells (Figure 1B). Notably, although we observed similar levels of transduction efficiency and level of mCherry expression for all 3 constructs, Jurkat cells transduced with MHB10.3 LV expressed significantly lower levels of TCR at the cell surface when compared to cells transduced with either RAR5.3 or HA307 TCRs (Figure 1B-C). These data demonstrate that each LV is able to transfer TCR expression and that expression of the MHB10.3 TCR is more variable than that of the RAR5.3 and HA307 TCRs. Transduction efficiency of all 3 lentiviral constructs was also similar in wild-type Jurkat cells containing an endogenous TCR (Figure 1D) allowing us to test the function of our TCRs in T cells with and without an endogenous TCR. However, we were unable to specifically detect transgenic TCRs due to the unavailability of TCR allele-specific antibodies or pMHC-multimers.

To test function of transgenic TCRs, lentivirus-transduced Jurkat or J76 cells were co-cultivated with peptide-pulsed EBV transformed B-cells. Expression of CD69 was measured in order to quantify TCR functionality, gating on mCherry+ events (Figure 2A). Stimulation of HA307 and RAR5.3 TCRs with cognate peptide induced significant CD69 upregulation in both Jurkat and J76 cell lines.

These data confirm functional signaling by the introduced TCRs, irrespective of the presence or absence of an endogenous TCR (Figure 2B- C). In contrast, neither J76 nor wild-type Jurkat cells transduced with MHB10.3 responded to their wild-type cognate peptide, despite responding to the positive control stimulus (Figure 2D). Previous studies with the original MHB10.3 T cell clone had identified an altered peptide ligand (APL), ΔC19, with superagonist properties (Tree, unpublished data). When this APL was added, we observed a small but nonetheless significant upregulation of CD69 in both MHB10.3+ Jurkat and J76 cell lines (Figure 2D). Peptide dose-response curves generated for each TCR+ Jurkat cell line revealed a clear hierarchy of responsiveness, with HA307 Jurkat cells responding to lower concentrations of peptide than RAR5.3 Jurkat cells (Figure 2E). Once again, the MHB10.3 Jurkat cells did not respond to cognate wild-type peptide at any concentration tested, whereas they did respond in a weak but dose-dependent manner to the ΔC19 APL.

3.2 Generation of antigen-specific human Treg using lentiviral transduction.

To test the function of our transgenic TCRs in human Tregs, $CD4^{+}CD25^{hi}CD127^{lo}$ T cells were isolated from PBMCs by flow sorting (Supplementary figure 1A). Isolation of cells with this phenotype has previously been demonstrated to yield a population of Tregs enriched for FOXP3 expression and with potent regulatory function[29]. Following transduction, mCherry+ Tregs were isolated by flow sorting (Figure 3A) and expanded, through two or three rounds, producing between 50 and 100 million transgenic Tregs from an initial population of $0.5\text{-}1x10^{6}$ Tregs (example expansion profiles for each TCR are shown in Supplementary figure 3). We observed no significant difference in the transduction efficiency of Tregs or the level of mCherry expression in transduced cells between TCR LVs. The purity of the expanded transgenic Treg populations were similar to those observed in untransduced expanded Treg populations (data not shown) and were consistently >90% FOXP3+ (Supplementary figure 1).

In order to test the function of transgenic TCRs, transduced Tregs were incubated with HLA-compatible PBMCs and cognate peptide. After 16 hours, CD69 expression was quantified within the mCherry+ gate (Figure 3B). In agreement with data generated using Jurkat cell lines, stimulation of HA307 and RAR5.3-engineered Tregs in this manner induced significant CD69 upregulation ion response to cognate but not irrelevant peptide (Figure 3C-D, Supplementary figure 4), confirming the ability of these TCRs to deliver an activating signal in human Tregs. However, we observed no response to wild-type B13 peptide in MHB10.3 Tregs. Furthermore, in contrast to results produced with Jurkat cells, MHB10.3 Tregs did not respond to the APL ΔC19. This did not reflect an intrinsic defect in these cells since they upregulated CD69 as expected upon treatment with CytoStim, a positive control reagent that crosslinks the TCR with MHC molecules found on APC (Figure 3E). As CD69 may not represent the best marker of activation for Tregs, we also assessed expression of other molecules associated with Treg activation (CD137 and GARP). Expression of these additional markers showed the same pattern of responses observed with CD69 with no evidence of activation of MHB10.3 Tregs by cognate peptide.

Since APC present in a mixed population of PBMC may not provide optimal stimulation of Tregs, we next tested the ability of HLA-matched EBV transformed B cells to present wild-type B13 or ΔC19 peptides and thereby stimulate MHB10.3-engineered Tregs. Using this approach, a sub-population of the engineered cells upregulated CD69 expression in response to the ΔC19 peptide. This allowed for the isolation of peptide responsive (mCherry+ CD69+) and unresponsive (mCherry+ CD69-) MHB10.3-transduced Tregs by flow sorting (Figure 4A). These Treg populations were re-stimulated, expanded and re-tested for responsiveness using peptides presented by PBMCs. While the CD69- MHB10.3 Tregs remained unresponsive to both B13 and ΔC19 peptides, the CD69+ population retained the ability to respond to the ΔC19 peptide (Figure 4B), demonstrating that this approach could be used to enrich antigen-responsive transgenic Tregs.

3.3 Antigen-specific suppression by TCR transgenic Treg.

To examine the ability of each transgenic Treg population to suppress T cells in an antigen-specific manner, we designed a suppression assay that examined both polyclonal and antigen-specific suppression. CellTrace Violet labeled PBMCs were stimulated with recall antigens leading to T cell proliferation and dye dilution (example gating strategy shown in Figure 5A). This proliferative CD4 and CD8 T cell response was measured in the presence of diluent or peptide to control for any effect the peptide had on T cell proliferation. Suppression was then measured at various ratios of transgenic Tregs to PBMCs (Figure 5B and 6).

Each transgenic Treg cell line suppressed both CD4 and CD8 proliferation in the absence of cognate peptide, indicating the presence of non-specific suppression (Figure 6A-C). This suppression was dependent upon dose of Tregs and, for CD4 responses, was negligible at PBMCs:Tregs ratios of 50:1 or lower. Stimulation of HA307 Tregs with cognate peptide resulted in a substantial enhancement of their suppressive activity that was evident even at very low ratios of PBMCs:Tregs (such as 1000:1), demonstrating the presence of potent antigen-specific suppression (Figure 6A). Similarly, we observed increased suppression when RAR5.3 Tregs were stimulated with cognate peptide. For example, at a ratio of 50:1, 1% of CD4+ T cells were suppressed in the absence of peptide while 25% of CD4+ T cells were suppressed in the presence of RAR5 peptide (Figure 6B). Since initial experiments with MHB10.3 Tregs showed no additional suppression when Tregs were stimulated with either wild-type B13 peptide or the APL ΔC19 (data not shown), we isolated a sub-population of MHB10.3 Treg based on the responsiveness to ΔC19 peptide presented by EBV transformed B cells as described above. Using this Treg population, we observed increased suppression in response to both ΔC19 APL and wild-type B13 peptide, although suppression was more potent when using the APL (Figure 6C). For example, at a ratio of 50:1, 12% suppression of CD4+ T cell proliferation was observed in the absence of peptide. This suppression increased to 30% upon addition of the wild-type B13 peptide, while addition of the APL ΔC19 increased suppression further to 54%. We quantified the

contribution of true antigen-specific suppression for each Treg cell line by subtracting the percentage suppression seen in the absence of stimulation from that seen in transgenic Treg-stimulated cultures (Figure 6D). This analysis confirmed that activation of each Treg cell line upon peptide recognition by their transgenic TCR induced antigen-specific suppression.

4. Discussion

Numerous studies in recent years have demonstrated that the ability of Tregs from individuals with T1D to suppress autologous effector T cell responses is reduced compared to non-diabetic individuals[3, 4, 30, 31]. Research addressing the cellular basis of this defective regulation has yielded two important insights. Firstly, although effector T cells from individuals with T1D are more resistant to regulation than those from non-diabetic individuals, this resistance is not absolute, and increasing the ratio of Tregs to effector T cells results in more effective suppression[7, 31]. Secondly, although Tregs do appear to be less potent in at least some individuals with T1D when analyzed directly *ex vivo*, expansion of these cells *in vitro* under optimal conditions results in Tregs with similar potency to those derived from non-diabetic individuals[29]. These findings highlight the potential for augmenting Treg function to treat T1D, a view supported by data from a number of clinical interventions that slowed β-cell destruction such as Teplizumab (anti-CD3)[32], Alefacept (LFA-3 Ig)[33] and anti-thymocyte globulin/G-CSF[34], which, although not originally designed to specifically target Tregs, were associated with a relative increase in the frequency of Tregs.

Based on these findings and promising results using polyclonal Treg therapy in the NOD mice[35], clinical trials have been initiated using Treg infusion to boost Treg numbers in individuals with T1D[12-14]. Large-scale expansion and infusion of autologous Tregs has been shown to be safe, with some efficacy reported in newly diagnosed children. While polyclonal Treg therapy is still in the early stages of clinical testing, data from animal models of transplant tolerance and T1D suggests that islet-antigen specific Tregs may be a more efficacious therapy, with 10-100 fold fewer cells required to prevent disease and a unique ability to reverse established hyperglycemia[15, 16, 18, 36].

As adoptive transfer of islet-antigen specific Tregs is predicted to be a more efficacious treatment for T1D, we sought to test whether LV TCR gene transfer into polyclonal Treg could produce populations of islet antigen-specific Tregs large enough to be used as a therapy. Current clinical protocols suggest that

infusion of $1x10^9$ polyclonal Tregs is required to produce a therapeutic effect in children newly diagnosed with T1D[37]. Based on data from the NOD mice, we predict infusion of 10-100 fold fewer islet antigen-specific Tregs would be capable of producing at least an equivalent, potentially greatly enhanced, therapeutic benefit. Our data indicates that we are able to routinely produce >100 million transgenic Tregs from a single blood draw of 100 ml, demonstrating the feasibility of our protocols for the production of clinically relevant numbers of islet antigen-specific Tregs. Given the known ability of FOXP3 Tregs to mediate linked suppression following activation via their TCR, we would suggest that a transgenic Treg specific for a single islet antigen would be able to mediate suppression of a wide range of effector T cells with specificity for a range of islet specific antigens present in the same anatomical location.

While our study demonstrates that TCR gene transfer can be used to produce islet antigen-specific Tregs, our results obtained using both Jurkat cells and primary human Tregs illustrate a hierarchy of TCR signaling capabilities, both in terms of markers of activation (CD69) and the ability to mediate antigen-specific suppression. The viral-specific HA307 TCR proved to be the most effective, followed by the islet-specific RAR5.3 TCR and finally the islet-specific MHB10.3 TCR. The latter required the use of an APL, a more potent APC population and further enrichment of responsive cells in order to produce Tregs capable of mediating antigen-specific suppression. In part, poor performance of the islet-specific TCRs could have resulted from variable levels of TCR expression as was observed for the MHB10.3 TCR. This issue could be overcome by the addition of a second disulphide bond between the TCR constant regions[38-40], or codon optimisation of the TCR genes[41, 42].

In addition to TCR expression, TCR affinity is also likely to be an important contributing factor to the suppressive potency of a transgenic Treg population. Investigation into how efficiently different TCRs activate Tregs has previously been performed using alloantigen-specific TCRs[43]. Comparison of two different TCRs with differing affinities but the same peptide specificity showed that Tregs transgenically expressing the higher affinity TCR were more effective

at promoting graft survival in a mouse model of transplantation compared to Tregs expressing the lower affinity TCR, suggesting that TCR affinity contributes to Treg function. In contrast, *Plesa et al.* observed a similar level of antigen-specific suppression in Tregs transfected with a high affinity HIV-specific TCR and a lower affinity tumor antigen-specific TCR[22]. However, it should be noted that the study was performed using MHC class I-restricted TCRs, which may have different signaling requirements and, as discussed below, both of these TCRs might be expected to have higher affinity than the autoreactive TCRs used in the present study. Recent data also suggests that signaling down-stream of TCR activation is diminished in Tregs in comparison to effector T cells[44], suggesting that Tregs may need TCRs with higher affinity in order to respond to a given peptide specificity when compared to effector T cells. Although we have not measured the affinities of the TCRs characterised in this study we can make predictions about their affinities based on the cell type from which they were derived. The autoreactive TCRs are derived from cells similar to Tr1 type regulatory T cells, which are likely to develop from the naïve T cell pool, the same pool from which autoreactive effector T cells are derived. Therefore, each TCR has survived negative selection in the thymus, suggesting that their affinities for autoantigen are low. In contrast, the HA307 TCR was derived from a Th1 clone and would likely have been selected on the basis of a high affinity for pathogen-derived peptides. The lower affinity of autoreactive TCRs could also be affected by differences in their docking topology with the MHC peptide complex. A recent study characterising the TCR crystal structures of two low affinity insulin-specific induced Treg clones discovered that both TCRs adopted a reversed docking topology atop the MHC complex when compared to a higher affinity pathogen-specific clone[45].

Overall, our data highlights that a more appropriate source of TCRs may be required when developing autoantigen-specific Treg therapies. TCR engineering could be employed to affinity mature the autoreactive TCRs used in this study into higher affinity variants; however, safety concerns due to potential off-target specificities make this option unattractive. Alternatively, naturally occurring islet-specific Tregs could be tested as a source of potentially higher affinity TCRs. Whether the TCRs from the CD4+CD25^{hi}CD127^{lo} Treg

population have similar characteristics to the autoreactive TCRs described here or are more comparable pathogen-specific TCRs would be an important question to answer for the development of TCR gene therapy for T1D.

5. Conclusions

This study demonstrates that human islet-antigen specific Tregs can be generated by lentiviral TCR gene transfer. TCR-transgenic Treg populations can be expanded to numbers compatible with testing in the clinical setting and retain their FOXP3 expression and suppressor function. This study provides proof-of-principle data for the use of islet antigen-specific TCRs in developing adoptive cell therapies for T1D, whilst highlighting that further optimisation of TCRs for use in such therapies may be required.

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Table 1. Allele usage of CD4+ T-cell clone derived TCR chains, identified by analysis of sequencing results using the IMGT database. For each TCR chain three pGEM-T plasmids were sequenced. Sequences were aligned and entered into the IMGT database to confirm that the sequence represented a successfully rearranged TCR chain (e.g. did not have any truncations and could produce a functional protein) and to identify which TCR α or β chain allele was present. IA-2 – islet antigen 2; Ins – insulin; HA – influenza virus hemagglutinin.

Figure 1. Expression of transgenic TCRs in model T cell lines using lentiviral expression constructs. Each TCR was expressed using a lentiviral vector. LTR – long terminal repeat (A). Stoichiometric expression of the TCR α and β chains along with the fluorescent marker mCherry red fluorescent protein was achieved using two intervening P2A peptides, each placed downstream of a furin cleavage site. Upon lentiviral infection of TCR $\alpha\beta$–ve J76 Jurkat cells, expression of the transgenic TCR and mCherry was assessed by flow cytometry (B). Mean fluorescence intensity (MFI) for both TCR staining and mCherry expression was compared for each cell line. Data shown is from three independent transductions (mean $\pm$SEM) (C). TCR+ve Jurkat cells were also infected with the indicated lentiviral constructs and expression of the TCR inferred using mCherry expression (D).

Figure 2. Validation of transgenic TCRs in model T cell lines. Islet- or influenza-specific TCRs were expressed with Jurkat or TCR-deficient J76 cells by lentiviral transduction. Transduced cells were cultured with EBV transformed B cells derived from donors with compatible MHC class II genotypes in the presence of peptide diluent (negative control), cognate or super-agonist peptide (at 10 μM), or CytoStim (positive control). Following incubation for 16 hours, cells were harvested and surface expression of CD69 was determined by flow cytometry, gating within the mCherry+ cell population. An illustrative example of this approach is shown (A). Co-cultures of EBV transformed B cells and TCR transgenic Jurkat cell lines either with (closed bars) or without an endogenous TCR (J76 cells; open bars) were established as described above. Percentage expression of CD69 was then calculated under each stimulation condition for

the HA307 (B), RAR5.3 (C) and MHB10.3 (D) cell lines (mean ±SEM). Statistical significance was determined using a two-tailed Student's *t* test and at least triplicate independent measurements. Each TCR+ve Jurkat cell line was then stimulated with titrated concentrations of the indicated peptide and expression of CD69 analyzed as described above (graph shows data from at least duplicate measurements and is representative of two independent experiments (mean ±SEM)) (E). Note that for the MHB10.3 TCR, B13-30 represents cognate wild-type peptide while ΔC19 is a super-agonistic peptide for this TCR.

Figure 3. Production and characterisation of transgenic human Treg. FACS purified human Treg were transduced with each TCR LV and isolated by flow sorting on the basis of mCherry expression. The percentage of transduced Tregs in each population is indicated (A). Following expansion, each Treg population was incubated with PBMC from donors with compatible MHC class II genotypes and peptide diluent (negative control), cognate or super-agonist peptide (at 10 μM), or CytoStim (positive control). Following incubation for 16 hours, cells were harvested and surface expression of CD69 by Tregs was determined by flow cytometry. An illustrative example is shown (B). Each stimulation condition was established in triplicate, analyzed independently and used to calculate mean percentage expression of CD69 for HA307 (C), RAR5.3 (D) and MHB10.3 (E) cell lines. Data shown is from triplicate independent measurements (mean ±SEM) and statistical significance was determined using a two-tailed Student's *t* test. Data are representative of two independent experiments.

Figure 4. Isolation of a functional MHB10.3 Treg population. MHB10.3 Treg were stimulated with the super-agonist ΔC19 peptide presented by EBV transformed B cells. Following a 16-hour incubation, cultures were stained for CD69 expression and CD69+ and CD69-, Cherry+ Tregs were isolated by flow sorting using the indicated gates (A). CD69+ and CD69- Tregs were expanded and the responses of each population to cognate and super agonist peptide was determined in independent triplicate cultures (B). Statistical significance

was determined using a two-tailed Student's *t* test. Data are representative of two independent experiments.

Figure 5. Example of antigen-specific suppression assay. CellTrace Violet labeled PBMCs from an HLA compatible individual were stimulated with the pentameric vaccine Pediacel at a final dilution of 1 in 1000 to induce a recall response in the presence or absence of 1 transduced Treg per 50 PBMCs alone or together with 10 μM peptide. This recall response was measured by CellTrace violet dye dilution within the CD4+ and CD4- T cell populations. Following a 7-day incubation period, triplicate wells were pooled and stained for CD3, CD4 and 7-AAD (to exclude dead cells). Lymphocytes were gated on the basis of FSC-A and SSC-A, doublets removed and live T cells gated on the basis of CD3 and 7-AAD staining. Within the live T cell gate, transgenic Tregs were excluded on the basis of high CD4 and mCherry expression (A). Percentage CellTrace Violet dye dilution was then assessed within the CD4+ (CD4+ T cells) and CD4- gates (CD8+ T cells) (B).

Figure 6. Antigen-specific suppression by TCR transgenic Tregs. PBMCs were stimulated with recall antigens Pediacel or Aggripal. Transgenic Tregs were then added to PBMC at the indicated ratios. Two different co-cultures were set up for each ratio, one stimulated further with the peptide to which the transgenic TCR is specific and one stimulated with the peptide's diluent control. At day 6, cultures were harvested and analyzed with % suppression calculated as follows: 100 – ((% proliferation in the presence of transgenic Tregs / % proliferation in the absence of transgenic Tregs)*100)
Suppression by HA307 Tregs (A), RAR5.3 Tregs (B), and CD69+ MHB10.3 Tregs (C) is shown). The change in suppression of CD4+ T cell proliferation upon peptide stimulation compared to diluent alone (Δ % suppression) is shown in (D). Statistical significance was determined using a two-tailed Student's *t* test. Graphs show triplicate independent measurements (mean ±SEM) and are representative of two independent experiments.

Supplementary figure 1. TCR nucleotide sequences. HA307 (A), RAR5.3 (B), MHB10.3 (C).

Supplementary figure 2. Isolation and phenotyping of human Tregs. Freshly isolated human PBMCs were stained with fluorescently labeled antibodies specific for CD4, CD127 and CD25. Lymphocytes were identified on the basis of forward and side scatter and doublets excluded. CD4+ T cells were then gated and within this population the CD127 low/negative CD25 high population was isolated as Treg (A). Following *in vitro* expansion, cells were rested for 48 hours in the absence of TCR stimulation or exogenous IL-2 before being characterised for expression of CD3, CD4, CD25 and FOXP3 compared to a population of expanded effector T cells (CD4+ CD25lo CD127hi T cells) (B and C).

Supplementary figure 3. Expansion profiles of transgenic Tregs. Cell number was monitored to test whether transduced Tregs could be expanded. Expansion of HA307 Tregs, is shown in (A), RAR5.3 Tregs in (B) and MHB10.3 Tregs in (C). Dotted lines indicate the day on which transgenic Tregs were isolated using mCherry expression and re-activated or cryopreserved.

Supplementary figure 4. Peptide responses of TCR transgenic Tregs. Upregulation of alternative Treg activation markers CD137 and GARP by transgenic Tregs in response to peptide was measured. Each Treg population was incubated with PBMC from donors with compatible MHC class II genotypes and peptide diluent (negative control), cognate or super-agonist peptide (at 10 μM), or CytoStim (positive control). Following incubation for 16 hours, cells were harvested and surface expression of CD69 by Tregs was determined by flow cytometry. An illustrative example is shown (B). Each stimulation condition was established in triplicate, analyzed independently and used to calculate mean percentage expression of CD69 for HA307 (C), RAR5.3 (D) and MHB10.3 (E) cell lines. Data shown is from triplicate independent measurements (mean ±SEM) and statistical significance was determined using a two-tailed Student's *t* test.

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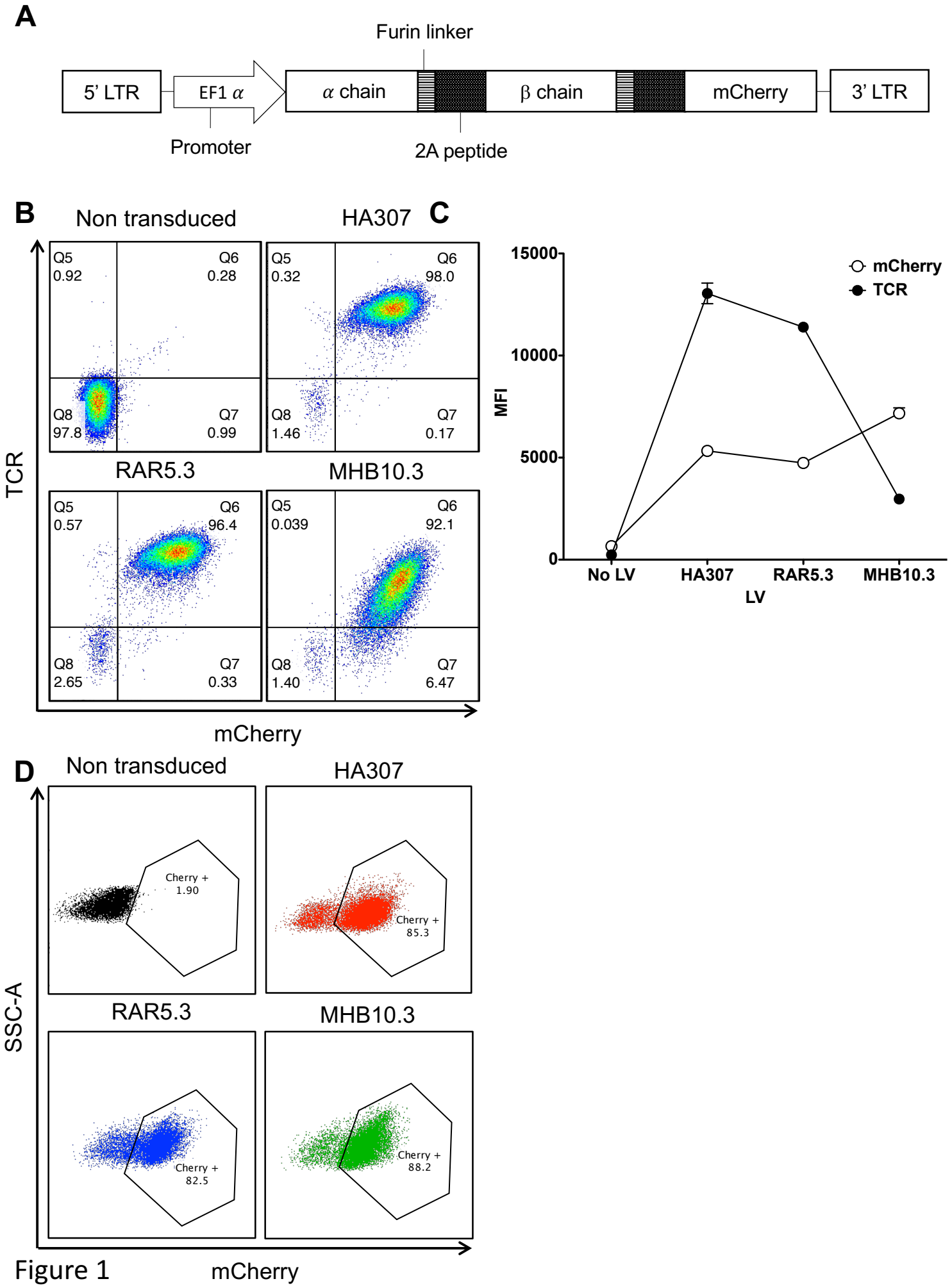
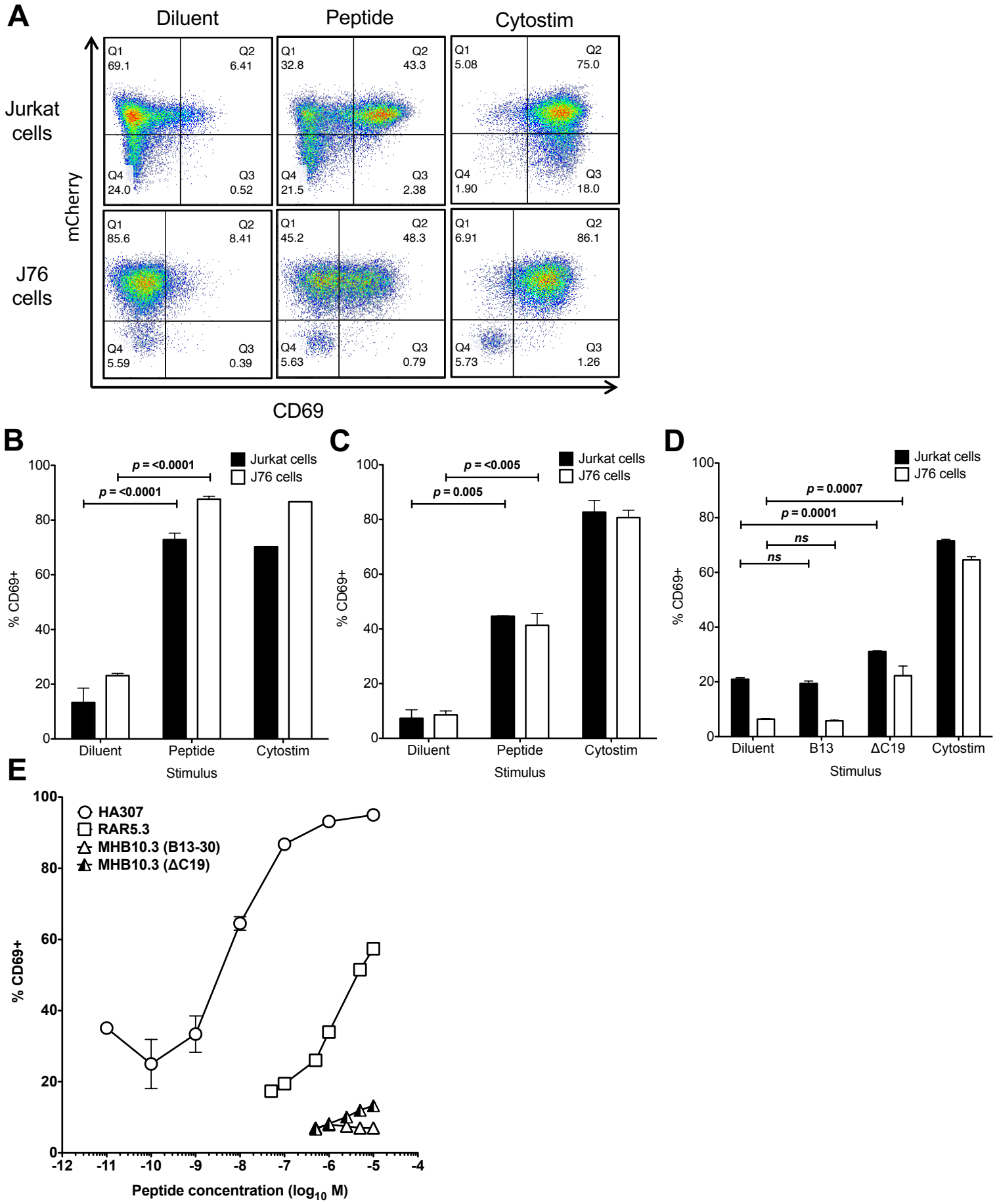

A
Furin linker
5' LTR
EF1 α
α chain
β chain
mCherry
3' LTR
Promoter
2A peptide
B
Non transduced
HA307
C
Q5
0.92
Q6
0.28
Q8
97.8
Q7
0.99
Q5
0.32
Q6
98.0
Q8
1.46
Q7
0.17
RAR5.3
MHB10.3
Q5
0.57
Q6
96.4
Q8
2.65
Q7
0.33
Q5
0.039
Q6
92.1
Q8
1.40
Q7
6.47
TCR
mCherry
15000
10000
5000
0
MFI
mCherry
TCR
No LV
HA307
RAR5.3
MHB10.3
LV
D
Non transduced
HA307
Cherry +
1.90
Cherry +
85.3
RAR5.3
MHB10.3
Cherry +
82.5
Cherry +
88.2
SSC-A
mCherry

Figure 1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Peptide
Cytostim
Jurkat cells
J76 cells
mCherry
CD69
Q1 69.1
Q2 6.41
Q4 24.0
Q3 0.52
Q1 32.8
Q2 43.3
Q4 21.5
Q3 2.38
Q1 5.08
Q2 75.0
Q4 1.90
Q3 18.0
Q1 85.6
Q2 8.41
Q4 5.59
Q3 0.39
Q1 45.2
Q2 48.3
Q4 5.63
Q3 0.79
Q1 6.91
Q2 86.1
Q4 5.73
Q3 1.26
B
% CD69+
Jurkat cells
J76 cells
p = <0.0001
p = <0.0001
Diluent
Peptide
Cytostim
Stimulus
C
% CD69+
Jurkat cells
J76 cells
p = <0.005
p = 0.005
Diluent
Peptide
Cytostim
Stimulus
D
% CD69+
Jurkat cells
J76 cells
p = 0.0007
p = 0.0001
ns
ns
Diluent
B13
ΔC19
Cytostim
Stimulus
E
% CD69+
HA307
RAR5.3
MHB10.3 (B13-30)
MHB10.3 (ΔC19)
Peptide concentration (log10 M)

Figure 2

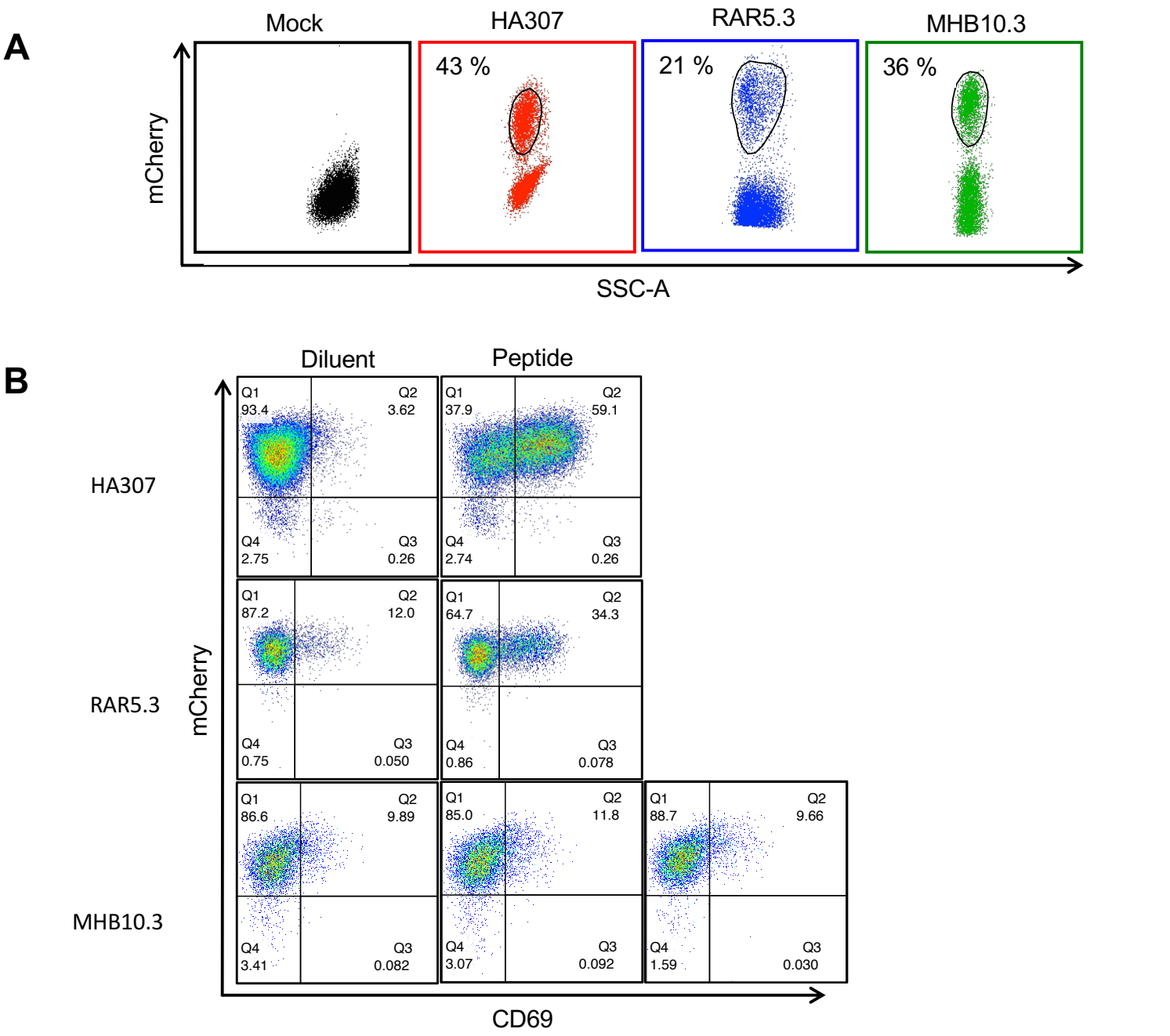

A
Mock
HA307
RAR5.3
MHB10.3
43 %
21 %
36 %
mCherry
SSC-A
B
Diluent
Peptide
HA307
RAR5.3
MHB10.3
mCherry
CD69
Q1 93.4
Q2 3.62
Q4 2.75
Q3 0.26
Q1 37.9
Q2 59.1
Q4 2.74
Q3 0.26
Q1 87.2
Q2 12.0
Q4 0.75
Q3 0.050
Q1 64.7
Q2 34.3
Q4 0.86
Q3 0.078
Q1 86.6
Q2 9.89
Q4 3.41
Q3 0.082
Q1 85.0
Q2 11.8
Q4 3.07
Q3 0.092
Q1 88.7
Q2 9.66
Q4 1.59
Q3 0.030
C
p = 0.0031
% CD69+
Diluent
Cytostim
HA307
D
p = 0.0002
% CD69+
Diluent
Cytostim
RAR5
E
ns
ns
% CD69+
Diluent
Cytostim
B13
ΔC19

Figure 3

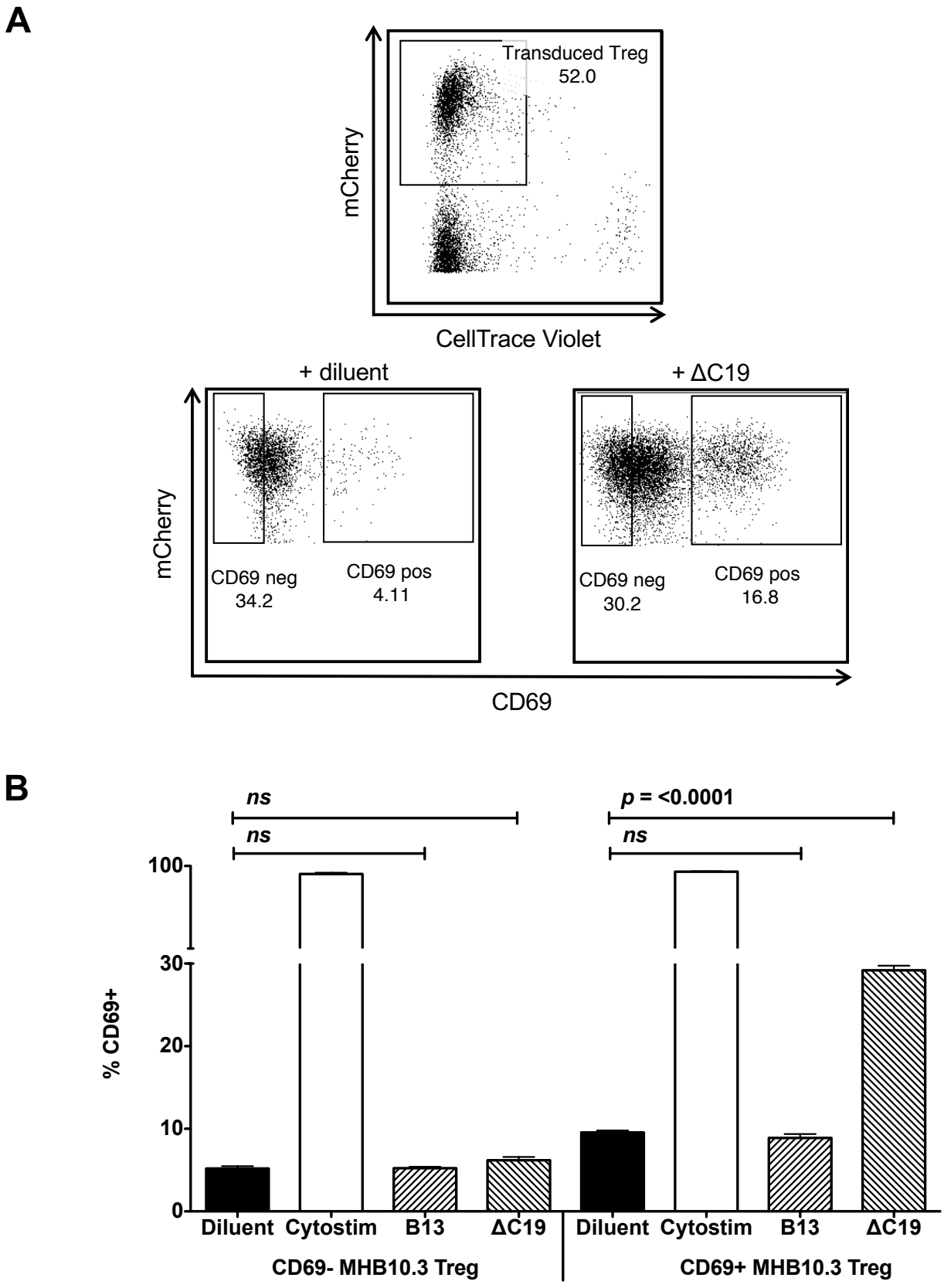
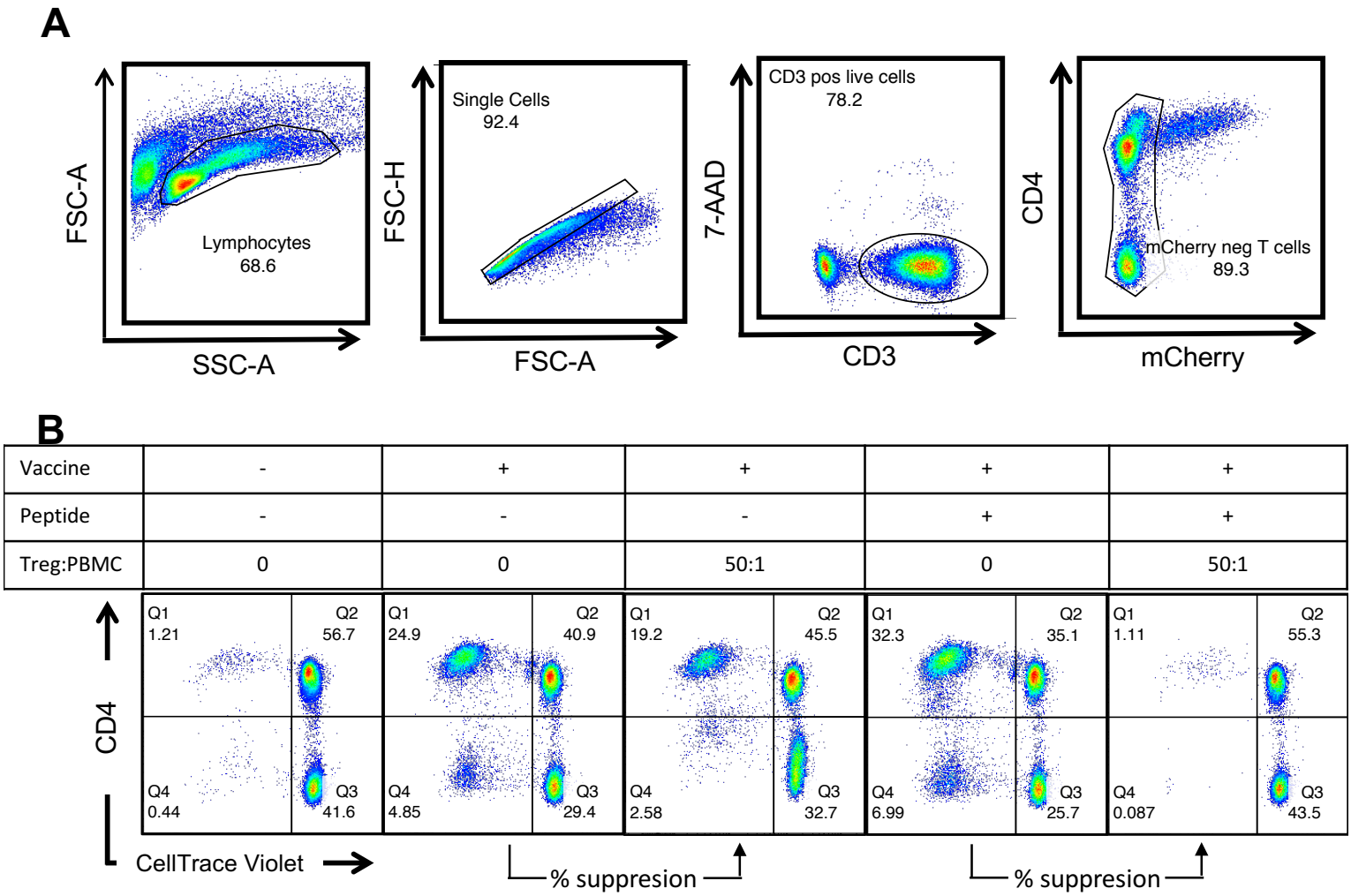

A
Transduced Treg
52.0
mCherry
CellTrace Violet
+ diluent
+ ΔC19
mCherry
CD69 neg
34.2
CD69 pos
4.11
CD69 neg
30.2
CD69 pos
16.8
CD69
B
ns
ns
p = <0.0001
ns
100
30
20
10
0
% CD69+
Diluent
Cytostim
B13
ΔC19
Diluent
Cytostim
B13
ΔC19
CD69- MHB10.3 Treg
CD69+ MHB10.3 Treg

Figure 4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FSC-A
SSC-A
Lymphocytes
68.6
FSC-H
FSC-A
Single Cells
92.4
7-AAD
CD3
CD3 pos live cells
78.2
CD4
mCherry
mCherry neg T cells
89.3
B
Vaccine - + + + +
Peptide - - - + +
Treg:PBMC 0 0 50:1 0 50:1
CD4
CellTrace Violet
Q1 1.21
Q2 56.7
Q4 0.44
Q3 41.6
Q1 24.9
Q2 40.9
Q4 4.85
Q3 29.4
Q1 19.2
Q2 45.5
Q4 2.58
Q3 32.7
Q1 32.3
Q2 35.1
Q4 6.99
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Q1 1.11
Q2 55.3
Q4 0.087
Q3 43.5
% suppresion
% suppresion

Figure 5

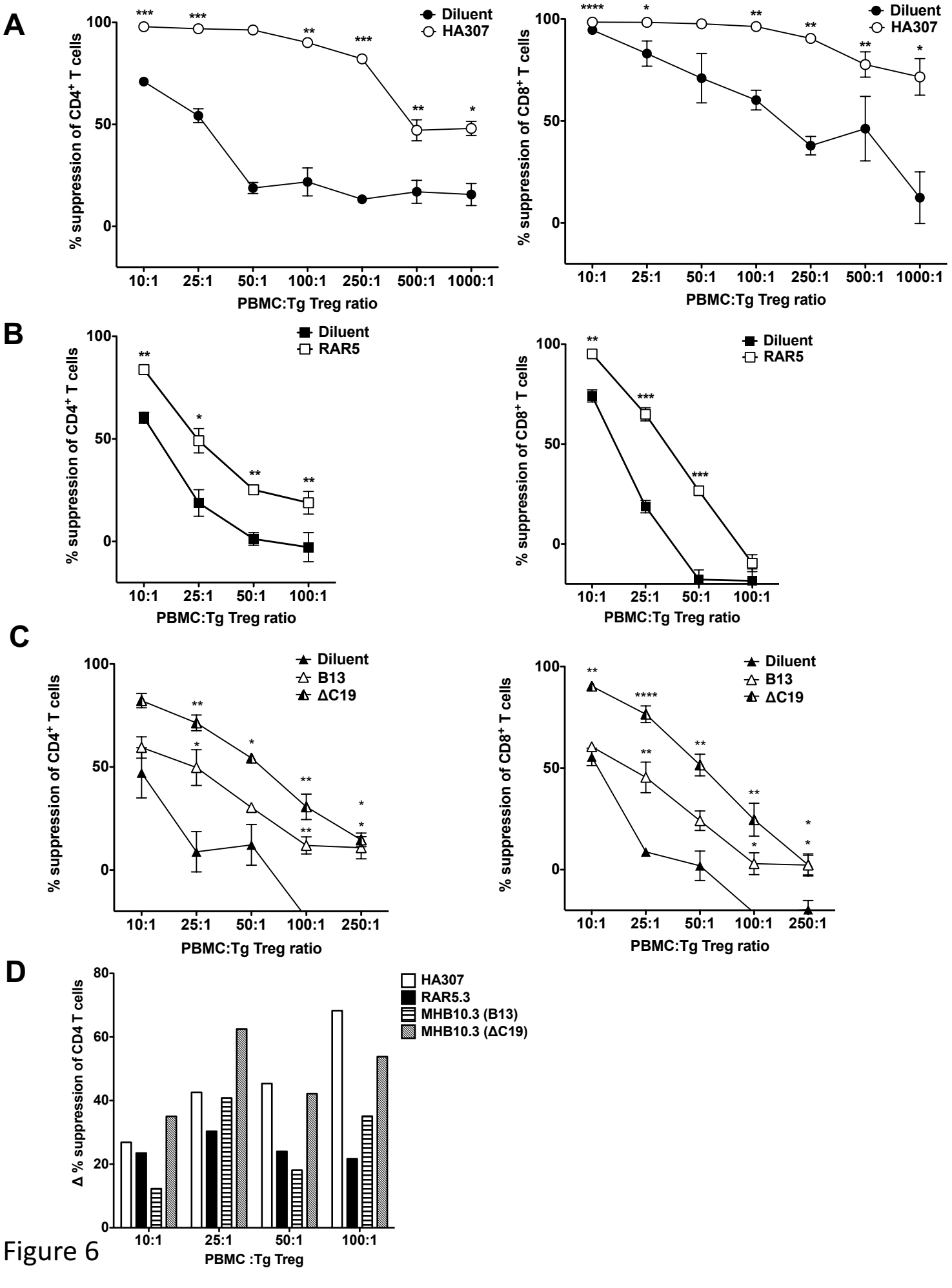
A
% suppression of CD4+ T cells
Diluent
HA307
10:1
25:1
50:1
100:1
250:1
500:1
1000:1
PBMC:Tg Treg ratio
% suppression of CD8+ T cells
B
RAR5
C
B13
ΔC19
D
Δ % suppression of CD4 T cells
HA307
RAR5.3
MHB10.3 (B13)
MHB10.3 (ΔC19)
PBMC :Tg Treg

Figure 6

HA307 TCR insert

ATGGCCATGAAATCCTTGAGAGTTTTACTAGTGATCCTGTGGCTTCAGTTGAGCTGGGTTTGGAGCCAACAGAAGGAGGTGGAGCAGAATTCTGGACC
CCTCAGTGTTCCAGAGGGAGCCATTGCCTCTCTCAACTGCACTTACAGTGACCGAGGTTCCCAGTCCTTCTTCTGGTACAGACAATATTCTGGGAAAAG
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ACCTTGTATGCCGTGCTGGTCAGTGCCCTCGTGCTGATGGCCATGGTCAAGAGAAAGGATTCCAGAGGCTAG

RAR5.3 TCR insert

ATGGCCATGCTCCTGGGGGCATCAGTGCTGATTCTGTGGCTTCAGCCAGACTGGGTAAACAGTCAACAGAAGAATGATGACCAGCAAGTTAAGCAAA
ATTCACCATCCCTGAGCGTCCAGGAAGGAAGAATTTCTATTCTGAACTGTGACTATACTAACAGCATGTTTGATTATTTCCTATGGTACAAAAAATACCC
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ATGGCCATGGTCAAGAGAAAGGATTCCAGAGGCTAG

MHB10.3 TCR insert

ATGAGGCAAGTGGCGAGAGTGATCGTGTTCCTGACCCTGAGTACTTTGAGCCTTGCTAAGACCACCCAGCCCATCTCCATGGACTCATATGAAGGACA
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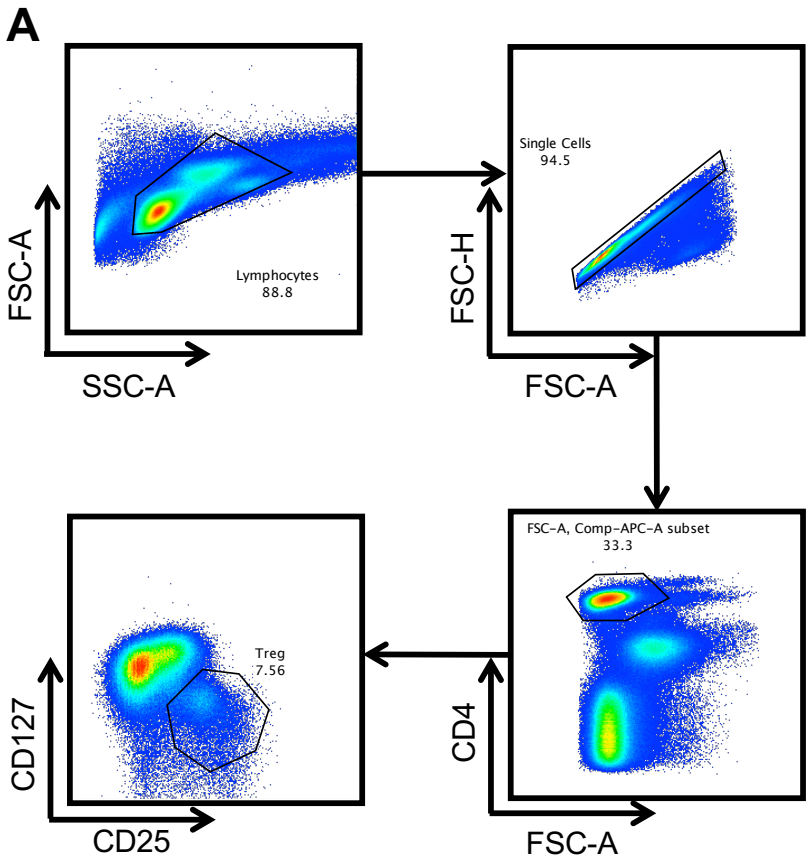
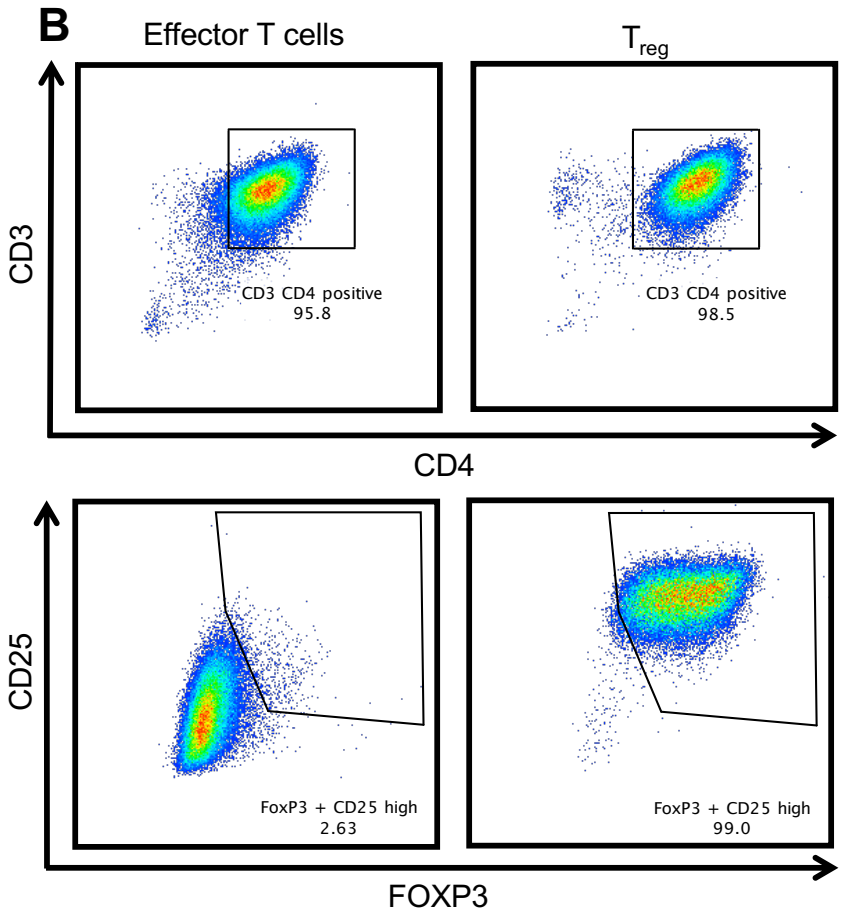
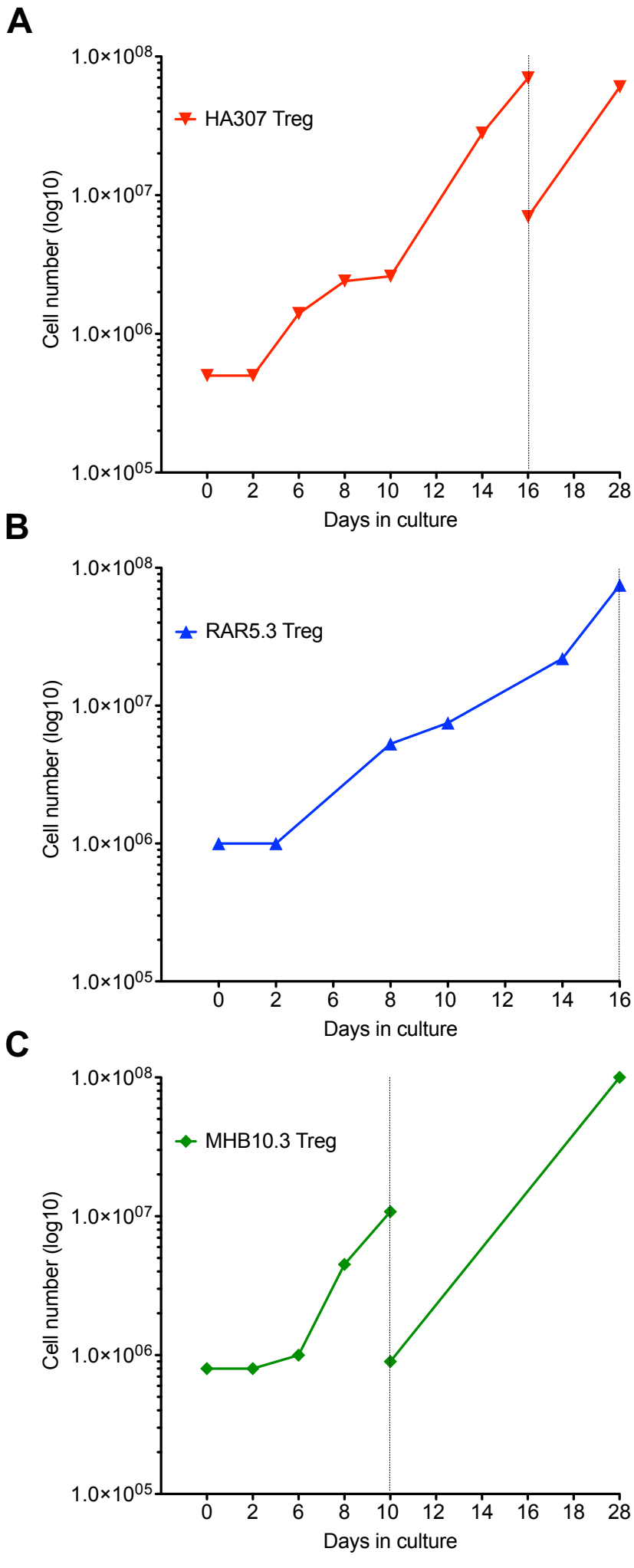
SF 1

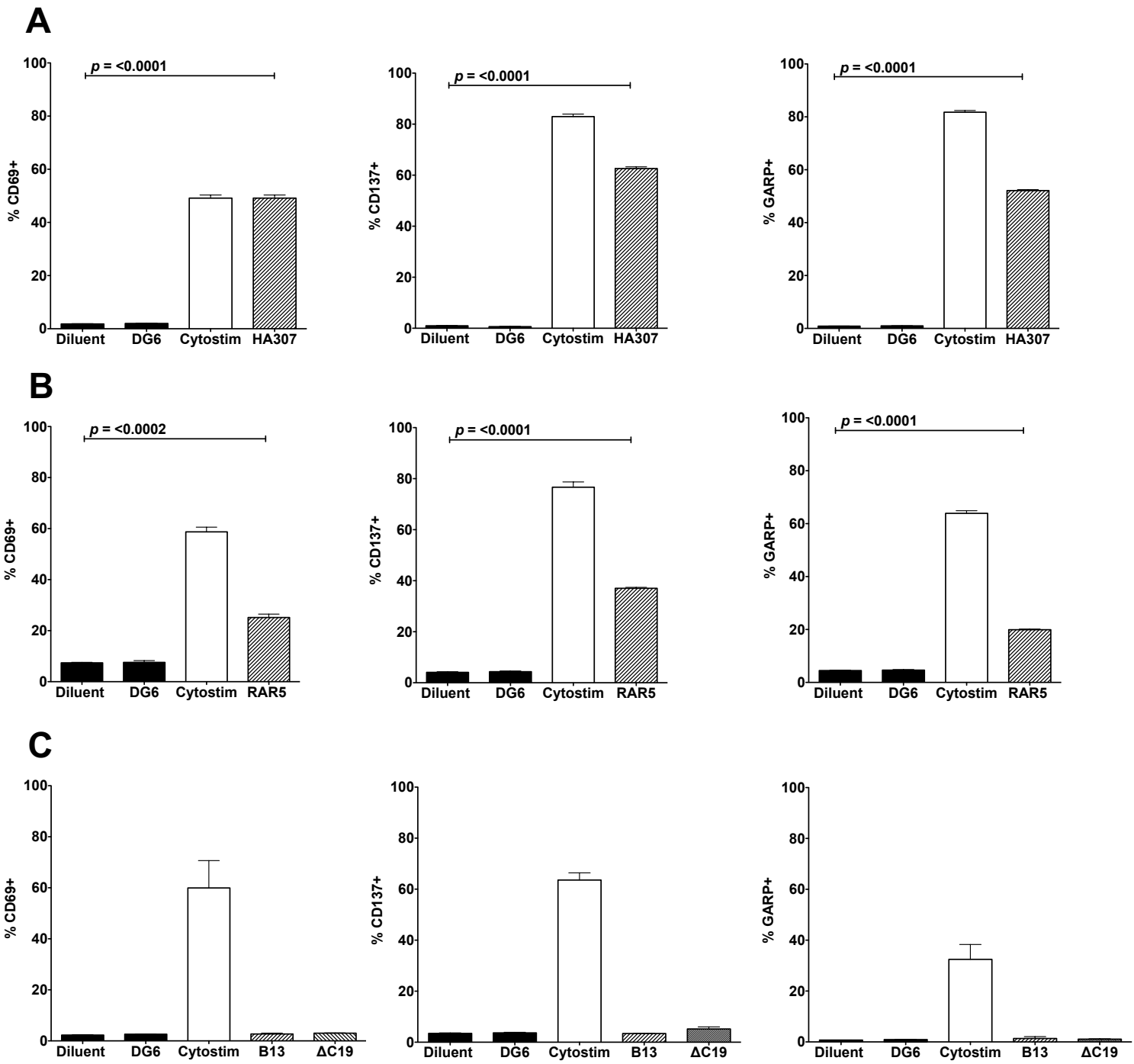

A
FSC-A
SSC-A
Lymphocytes
88.8
Single Cells
94.5
FSC-H
FSC-A
FSC-A, Comp-APC-A subset
33.3
CD4
FSC-A
Treg
7.56
CD127
CD25


B
Effector T cells
Treg
CD3
CD3 CD4 positive
95.8
CD3 CD4 positive
98.5
CD4
CD25
FoxP3 + CD25 high
2.63
FoxP3 + CD25 high
99.0
FOXP3

SF 2


A
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
Cell number (log10)
HA307 Treg
0 2 6 8 10 12 14 16 18 28
Days in culture
B
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
Cell number (log10)
RAR5.3 Treg
0 2 6 8 10 12 14 16
Days in culture
C
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
Cell number (log10)
MHB10.3 Treg
0 2 6 8 10 12 14 16 18 28
Days in culture

SF 3


A
p = <0.0001
% CD69+
% CD137+
% GARP+
Diluent
DG6
Cytostim
HA307
B
p = <0.0002
p = <0.0001
RAR5
C
B13
ΔC19

SF 4